The effects of the oral, pan-VEGF-R kinase inhibitor CEP-7055 and chemotherapy in orthotopic models of glioblastoma and colon carcinoma in mice

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Abstract
CEP-7055, a fully synthetic, orally active N,N-dimethylglycine ester of CEP-5214, a C3-(isopropylmethoxy)-fused pyrrolocarbazole with potent pan-vascular endothelial growth factor receptor (VEGFR) kinase inhibitory activity, has recently completed phase I clinical trials in cancer patients. These studies evaluated the antitumor efficacy of CEP-7055 using orthotopic models of glioblastoma and colon carcinoma in combination with temozolomide, and irinotecan and oxaliplatin, respectively, for their effects on primary and metastatic tumor burden and median survival. Chronic administration of CEP-7055 (23.8 mg/kg/dose) and temozolomide resulted in improvement of median survival of nude mice bearing orthotopic human glioblastoma xenografts compared with temozolomide alone (261 versus 192 days, respectively; \( P \leq 0.02 \)). Reductions in neurologic dysfunction, brain edema, hemorrhage, and intratumoral microvessel density (CD34 staining) were observed in glioma-bearing mice receiving CEP-7055 alone, temozolomide alone, and the combination of CEP-7055 and temozolomide relative to vehicle and to temozolomide monotherapy. The administration of CEP-7055 in combination with irinotecan (20 mg/kg/dose i.p. \( \times 5 \) days), and to a lesser degree with oxaliplatin (10 mg/kg/dose i.v.), showed reductions on primary colon carcinoma and hepatic metastatic burden in the CT-26 tumor model relative to that achieved by irinotecan and oxaliplatin monotherapy. These data show the significant efficacy and tolerability of optimal efficacious doses of CEP-7055 when given in combination with temozolomide and irinotecan relative to monotherapy with these cytotoxic agents in preclinical orthotopic glioma and colon carcinoma models and lend support for the use of these treatment regimens in a clinical setting in patients with glioblastoma and colon carcinoma. [Mol Cancer Ther 2006;5(7):1744–53]

Introduction
Primary brain tumors and colon carcinoma represent two devastating cancers having unmet therapeutic need for novel treatments to improve patient survival. Malignant neoplasms of the central nervous system are the fifth most common primary tumor in the United States, with an average incidence of 5 to 10 cases per 100,000 population (1, 2). Approximately 50% of these brain tumors are astrocytomas, of which 50% are classified as highly vascularized glioblastoma multiforme (2, 3). Although it represents only 1% to 2% of all malignancies, glioblastoma multiforme is diagnosed in 15,000 to 20,000 patients yearly. Despite aggressive therapy entailing surgery, radiation, and chemotherapy, the median patient survival is \( \leq 1 \) year (4). Temozolomide, an orally active imidazo-trazinone prodrug, has shown promising, albeit limited, objective responses (9–13%) in clinical trials in patients with high-grade malignant gliomas and is currently the standard of care for the management of this tumor type (5–7). Temozolomide administration has been shown to improve patients’ quality-of-life, but limited objective response rates and lack of significant survival benefit using temozolomide monotherapy have prompted studies using temozolomide in combination with additional therapeutic approaches, including antiangiogenic agents (4, 7, 8). Colorectal cancer is the second leading cause of cancer deaths in the United States with an estimated 150,000 diagnosed cases and >56,000 fatalities from the disease (9). Approximately one third of cases are localized to the colon and rectum and have a favorable prognosis, whereas one third of patients present with regional lymph node metastases at diagnosis and generally are refractory to various chemotherapeutic regimens. For the past 30 years, the major therapeutic option for patients with colorectal carcinoma has been based almost exclusively on 5-fluorouracil alone or in combination with leucovorin. The addition of irinotecan or oxaliplatin to the 5-fluorouracil/leucovorin regimen has made a significant effect on response rates and progression-free survival in clinical trials (10–13). However, although these agents have greatly improved survival and/or time to progression of disease, new treatments are required to prevent local recurrences or distant metastases (14, 15).
Increased expression of vascular endothelial growth factors (VEGF) by tumor cells and associated VEGF receptors (VEGFR), VEGFR2/KDR and VEGFR1/FLT-1, by the tumor-associated vasculature are a hallmark for a variety of human and murine tumors in vivo and are correlated with tumor growth rate, microvessel density/ proliferation, tumor metastasis, and poor prognosis in a variety of malignancies (16–19). The role of VEGFR in the growth, progression, and invasiveness of astrocytic brain tumors, including glioblastoma multiforme, has become clear in the past decade (20–22). In view of the highly vascularized nature of glioblastoma multiforme, several antiangiogenic agents have been evaluated preclinically and clinically in this malignancy (23–25). Consequently, novel therapies directed at inhibiting the VEGFR kinase pathway may provide additional therapeutic options for managing primary tumor growth and invasiveness of glioblastoma multiforme. Similarly, in colon cancer, the overexpression of VEGF and its tyrosine kinase receptors correlates with the development of hepatic metastases and poorer prognosis (26, 27). A variety of antiangiogenic therapies are currently under clinical evaluation in combination with established chemotherapeutic regimens and may represent a viable treatment option for patients with advanced colorectal cancer (14, 15, 23–27).

In this report, we describe the effects of chronic administration of CEP-7055 (28, 29), the prodrug of CEP-5214, a low nanomolar, orally active inhibitor of all three VEGFR kinase receptor subtypes (VEGFR1/FLT-1 IC50, 16 nmol/L; VEGFR2/KDR IC50, 8 nmol/L; and VEGFR3/FLT-4 IC50, 4 nmol/L) alone and in combination with standard of care chemotherapeutic agents for their effects on primary and metastatic tumor burden and survival in two highly specialized and aggressive orthotopic tumor models in mice. CEP-7055 was evaluated in combination with temozolomide (Schering-Plough, Kenilworth, NJ) and irinotecan (Pharmacia & Upjohn, Kalamazoo, MI) in a human orthotopic glioblastoma multiforme mouse model previously described (28). Details of the pharmacology and pharmacokinetics of CEP-7055 have been described previously (28). For all in vivo experiments described using CEP-7055, the HCl salt was used at >97% purity. A dose of 1.19 mg/kg CEP-7055 prodrug is equivalent to 1.0 mg/kg dose of CEP-5214. The pharmacologic development of CEP-7055 is being pursued in the context of a partnership agreement between Cephalon and Sanofi-Aventis (Gentilly, France). In earlier studies, CEP-7055 was formulated in 1% aqueous acetic acid (28), temozolomide (Schering-Plough, Kenilworth, NJ) and irinotecan (Pharmacia & Upjohn, Kalazoo, MI) were resuspended in 0.9% sterile saline, and oxaliplatin (Sanofi-Aventis, New York, NY) was resuspended in 5% dextrose immediately before administration (11).

Cell Lines

U87MG human glioma cells (American Type Culture Collection, Manassas, VA) were cultured in MEM (Life Technologies, Grand Island, NY) with 10% FCS (HyClone, Logan, UT) and CT-26 murine colon carcinoma cells (provided by Dr. James L. Abbruzzese, University of Texas M. D. Anderson Cancer Center, Houston, TX) were cultured in RPMI 1640 (Life Technologies) with 10% FCS. The cell lines were MAP-16 and Mycoplasma tested (Bio Reliance Corp., Rockville, MD) and deemed suitable for in vivo studies.

Animals

Female athymic nu/nu mice (6–8 weeks old; Charles River, Wilmington, MA) were maintained five per cage in microisolator units on a standard sterilizable laboratory diet (Teklad Labchow, Harlan Teklad, Madison, WI). Animals were housed under humidity- and temperature-controlled conditions and the light/dark cycle was set at 12-hour intervals. Female BALB/c mice (6–8 weeks old; Charles River) were maintained five per cage on a standard laboratory diet (Teklad Labchow). Animals were housed under humidity- and temperature-controlled conditions and the light/dark cycle was set at 12-hour intervals. Mice were quarantined 1 week before experimental manipulation. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of Cephalon, Inc.

U87MG Human Glioblastoma Cell Implantation and Dosing Regimens

An orthotopic human glioma brain tumor model using the human U87MG glioma cell line was developed in nude mice using a modification of a protocol described previously (30, 31). Briefly, female nude mice (nu/nu) were anesthetized via i.m. injection of a mixture of ketamine HCl
and xylazine and maintained with isoflurane in a nose cone apparatus. Once a surgical plane of anesthesia was reached, the mice were positioned in a stereotactic frame and a small, midline scalp incision was made. A burr hole was made over the right cranial hemisphere at a position 1.2 mm posterior and 1.9 mm lateral to the bregma. Using a sterile 10 μL Hamilton syringe with a 26-gauge needle, 5 × 10^5 cells in 2.5 μL sterile 1× PBS were implanted 2.2 mm deep into the right cranial hemisphere near the caudate putamen over a 3-minute period. Following implantation of the cells, the needle was left in place for 5 minutes. Finally, the needle was slowly withdrawn from the brain over 4 minutes (1.0 mm/min) and the skin incision was closed with a sterile clip and tissue glue. All surgical procedures were done under a laminar flow hood using aseptic techniques.

The dosing regimens of temozolomide and CEP-7055 have been detailed previously (28, 32) and were used in two parallel studies. Three days following recovery from intracranial surgery, mice were randomized into the following treatment groups: CEP-7055 (23.8 mg/kg/dose p.o. b.i.d. in 1% aqueous acetic acid vehicle; n = 8), temozolomide (68 mg/kg/dose p.o. once daily for 5 days in 0.9% sterile saline; the 5-day dosing cycle was repeated at day 56; n = 10), temozolomide plus CEP-7055 in combination given at the dosages described above (n = 8), or vehicle (n = 9). In the second study were randomized into the following groups: CEP-7055 (23.8 mg/kg/dose p.o. b.i.d. in 1% aqueous acetic acid vehicle; n = 14), CEP-7055 (59 mg/kg/dose p.o. b.i.d. in 1% aqueous acetic acid vehicle; n = 15); temozolomide (68 mg/kg/dose p.o. once daily for 5 days in sterile saline, the 5-day dosing cycle was repeated at day 56; n = 15), temozolomide plus CEP-7055 in combination given at the 23.8 and 59 mg/kg doses described previously (n = 15), or vehicle (n = 15). In both studies, temozolomide was given by gavage 1 hour before CEP-7055 to mice receiving the combination therapy. The mice were monitored daily for signs of neurologic dysfunction as assessed by circling or twisting behaviors, tremors, and unresponsiveness and weighed twice weekly until the mice died or were euthanized. At the time of sacrifice, the mice were necropsied for gross assessment of local tumor burden and histopathologic analysis of the brain. The presence or absence of hemorrhagic lesions was defined following gross inspection of the brain tissue, and the extent and degree of grossly visible cerebral swelling (edema) was assessed using the following scoring system: 0, no visible tumor; 1, edematous lesions confined to surrounding tumor at implantation site; 2, edematous lesions spread into brain tissue adjacent to tumor implantation site; 3, edematous lesions identified in 3 of 4 (75%) quadrants of brain tissue; 4, edematous lesions identified in 4 of 4 (100%) quadrants of brain tissue. Specimens of the brain were formalin fixed, paraffin embedded, and sectioned for histopathologic analysis of invasive foci following H&E staining.

Immunohistochemical confirmation of human glioma cells in the brain and surrounding brain stroma were assessed in tissue sections for the presence or absence of the glial differentiation marker, glial fibrillary acidic protein, as detailed (33). In addition, evaluation of intratumoral microvessel density (factor VIII and CD34 immunostaining) in glioma lesions was conducted as described (28).

**CT-26 Murine Colon Carcinoma Cell Implantation and Dosing Regimens**

The CT-26 murine colon carcinoma metastasis model using the murine CT-26 colon carcinoma cell line was developed in BALB/c mice using a modification of a protocol described previously (27, 34). Briefly, female BALB/c mice were anesthetized with ketamine/xylazine and maintained with isoflurane in a nose cone apparatus. A small incision was made to the left lateral quadrant of the abdomen and CT-26 colon carcinoma cells (1×10^6 in 50 μL sterile 1× PBS) were injected into the splenic parenchyma. Abdominal incisions were closed with 6-0 Vicryl sutures and the skin incision closed with autoclips. Dosing regimens for irinotecan and oxaliplatin have been detailed previously (11, 12, 32, 35). Two days following recovery from surgery, mice were randomized into the following treatment groups (n = 10): 1% aqueous acetic acid vehicle, CEP-7055 monotherapy (23.8 mg/kg/dose p.o. b.i.d.), irinotecan 20 mg/kg/dose i.p. once daily for 5 days in 0.9% sterile saline, CEP-7055 in combination with irinotecan dosed as described above, oxaliplatin 10 mg/kg/dose i.v. in 5% dextrose, and CEP-7055 in combination with oxaliplatin dosed as described above. The irinotecan or oxaliplatin was given 1 hour before CEP-7055 administration for mice receiving the combination therapy. Mice were monitored daily for signs of morbidity and weighed twice weekly until the end of the study when vehicle-treated mice started to die (day 18 of dosing). At the time of sacrifice, the mice were necropsied for gross assessment of local and hepatic tumor burden and tissues were taken for histopathologic analysis.

**Statistical Analyses**

The effects of CEP-7055 and temozolomide alone and in combination on survival of tumor-bearing mice were analyzed by the Kaplan-Meier method as required for data sets using SAS version 8.2 (SAS Institute, Inc., Cary, NC). Mann-Whitney rank-sum test analyses were used to compare mean and median survival times between treatment groups. Effects of CEP-7055 and/or temozolomide, irinotecan, or oxaliplatin on the weight of primary tumors (brain or spleen), weight of liver with metastatic lesions, and histopathologic analysis of the brain were assessed by the Mann-Whitney rank-sum test, with V0.05 deemed significant. For quantitation of intratumoral microvessel density (factor VIII and CD34 immunostaining as detailed in ref. 28) were assessed by the Mann-Whitney rank-sum test, with P ≤ 0.05 deemed significant. For quantitation of intratumoral microvessel density, 10 fields (97,500 mm²/field) of five tumors were evaluated in a blinded fashion at ×100 magnification, and the percent inhibition of microvessel density relative to vehicle-treated tumors was determined as described previously (28). Percent reductions were determined by determining mean values for control and treated groups, subtracting the treated group mean value from control group mean value, dividing this value by the control group mean value and then multiplying by 100 to give percent difference.
Results

Effects of Oral Administration of CEP-7055 Alone and in Combination with Temozolomide on Primary Tumor Growth, Survival, and Neurologic Dysfunction in an Orthotopic Model of Human Glioblastoma in Nude Mice

The effects of the administration of CEP-7055 alone, temozolomide alone, and the combination of CEP-7055 with temozolomide on tumor burden, neurologic dysfunction, and survival were evaluated using a clinically relevant orthotopic human glioblastoma multiforme model in nude mice. The results of the initial study revealed that CEP-7055 monotherapy had no significant effect on median survival (33 days), whereas temozolomide monotherapy showed a significant improvement in the median survival time of tumor-bearing mice relative to vehicle-treated mice (212 versus 27 days, respectively; \( P < 0.0001 \)). The combination of CEP-7055 (23.8 mg/kg/dose) and temozolomide administration also had a significant effect on improving the median survival of tumor-bearing mice compared with vehicle controls (241 versus 27 days, respectively; \( P < 0.0001 \)). More importantly, the combination of CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) and temozolomide (56-day cycles of 68 mg/kg/dose p.o. once daily for 5 days) administration resulted in a statistically significant improvement in median survival of tumor-bearing mice relative to temozolomide monotherapy (241 versus 212 days, respectively; \( P \leq 0.05 \); Fig. 2A). Comparison of brain weights in the CEP-7055 monotherapy versus vehicle-treated tumor-bearing mice revealed no statistically significant differences, suggesting a marginal effect of CEP-7055 monotherapy on intracranial tumor mass. In contrast, brain weights in the CEP-7055 and temozolomide combination treatment mice and the temozolomide monotherapy mice revealed statistically significant decrease relative to vehicle control mice (\( P < 0.001 \) for both treatment groups), suggesting a significant effect of treatments on reducing intracranial tumor mass.

A second orthotopic glioblastoma study conducted in parallel confirmed the improved median survival benefit of the combination therapy of CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) and temozolomide (56-day cycles of 68 mg/kg/dose p.o. once daily for 5 days) compared with that achieved with temozolomide monotherapy (261 versus 192 days, respectively; \( P < 0.02 \)). Oral administration of higher doses of CEP-7055 (59 mg/kg/dose b.i.d.) did not confer an additional survival benefit (31 days for 23.8 mg/kg CEP-7055 monotherapy) in this tumor model (Fig. 2B). In both series of studies, the combination of both agents was well tolerated with no distinct drug-associated morbidity or toxicities observed with chronic administration over an ~400-day in-life phase of the studies.

Immunohistochemical analyses of brain tumors in nude mice were done using glial fibrillary acidic protein to confirm the glial nature of the tumor and the gross reduction in tumor mass and local invasiveness associated with CEP-7055 and temozolomide treatments. H&E photomicrographs in Fig. 3 reveal the differences in tumor morphology and invasiveness between the different treatment groups.

Intratumoral microvessel density was also evaluated as an indirect assessment of effects on tumor angiogenesis. The analysis revealed that CEP-7055 monotherapy (23.8 mg/kg/dose p.o. b.i.d.) resulted in a 43% reduction in microvessel density per field compared with tumors in vehicle control mice (\( P < 0.01 \)). Evaluation of microvessel
density was not feasible in the temozolomide and CEP-7055 combination groups due to limited tumor tissue availability. We have shown previously that treatment with CEP-7055 caused significant reductions in intratumoral microvessel density, proliferative fraction (Ki-67), and tumor apoptosis and necrosis in multiple s.c. tumor xenografts in mice, including the U87MG glioblastoma cell line (28).

Reductions in the severity or total number of mice exhibiting neurologic dysfunction (as assessed by circling or twisting behaviors, tremors, and unresponsiveness to stimuli) were seen in tumor-bearing mice receiving CEP-7055 alone, temozolomide alone, and the combination of CEP-7055 and temozolomide compared with vehicle-treated controls in both studies. Histologic analyses revealed that edematous and hemorrhagic lesions were absent in mice receiving CEP-7055 (23.8 and 59 mg/kg doses) and temozolomide combination therapy, consistent with the reduction in tumor mass, whereas chronic administration of temozolomide alone resulted in significant reductions in brain edematous and hemorrhagic lesions in orthotopic glioma-bearing mice but did not completely eliminate lesions. Administration of CEP-7055 alone at 23.8 and 59 mg/kg doses showed significant reductions in widespread brain edematous (scores of 3 and 4) and hemorrhagic lesions (P < 0.05) relative to vehicle controls (Table 1; Fig. 3). The reductions in edematous lesions and neurologic dysfunction (behavior) observed with CEP-7055 monotherapy, despite the absence of significant effects on intracranial tumor mass and median survival, are consistent with the pharmacologic activity of this dose of CEP-7055 on vascular permeability in rodents (28, 36). The histologic findings and the observed reduction in intracranial tumor mass in the temozolomide monotherapy and CEP-7055 and temozolomide combination treatment groups are consistent with the observed improvements in neurologic function observed in these mice relative to vehicle-treated controls.

Effects of Oral Administration of CEP-7055 Alone and in Combination with Irinotecan or Oxaliplatin on Primary Tumor Growth and Metastases in a Metastasis Model of Colon Carcinoma in BALB/c Mice

The effects of CEP-7055 administration alone and in combination with clinically based dosing regimens of irinotecan or oxaliplatin on primary colon tumor and hepatic metastatic burden were evaluated using the murine CT-26 orthotopic colon carcinoma model in BALB/c mice (Fig. 4A–C). Administration of CEP-7055 alone to CT-26 colon tumor-bearing mice had no significant effects on primary tumor mass relative to vehicle controls; however, irinotecan monotherapy (20 mg/kg/dose i.p. × 5 days) and oxaliplatin monotherapy (10 mg/kg/dose i.v.) resulted in a 71% (P < 0.001) and 53% (P < 0.05) reduction in primary tumor mass, respectively, relative to vehicle controls. The combination of irinotecan and CEP-7055, however, resulted in an 86% reduction in primary tumor mass relative to vehicle control mice (P < 0.001) and

Figure 3. Histopathology photomicrographs of orthotopic glioblastoma multiforme tumors in glioblastoma multiforme implanted nude mice given CEP-7055 and temozolomide. A, vehicle-treated larger tumor with central necrosis (Necr) and numerous blood vessels in the periphery of the tumor (arrows). The glioblastoma multiforme cells show a pleomorphic morphology with predominate spindle cell proliferation with some epithelioid and giant cells. B, CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) + temozolomide (88 mg/kg/dose p.o. q.d. × 5 d)–treated tumors; monomorphic epithelioid cellular pattern, absence of necrosis, and marginal angiogenesis (arrow). C, vehicle-treated tumor, irregular tumor brain interface (arrows), showing penetrating tumor cell islets as well as areas of hemorrhage (asterisks). D, temozolomide-treated, well-delineated tumor/brain interface (arrows) representing an expansive growth pattern without obvious invasion into the surrounding tissue. E, vehicle-treated tumor with numerous mitoses showing a pleomorphic/spindle-type astrocytoma/glioblastoma multiforme morphology. F, CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) + temozolomide (88 mg/kg/dose p.o. q.d. × 5 d)–treated, monomorphic epithelioid astrocytoma/glioblastoma multiforme xenograft exhibiting few mitoses. Note that this pattern reflects a higher degree of differentiation compared with vehicle-treated tumors (E).
Temozolomide: 68 mg/kg/dose

CEP-7055: 59 mg/kg/dose + temozolomide

Fig. 4A). In contrast, although the combination of oxaliplatin and CEP-7055 resulted in a significant reduction in primary tumor mass relative to vehicle controls (P < 0.05), there was no additional benefit of CEP-7055 and oxaliplatin given in combination relative to oxaliplatin monotherapy; a slight reduction in antitumor effect relative to that achieved with oxaliplatin monotherapy was actually observed (Fig. 4A).

The CT-26 colon model is widely used to evaluate effects of cancer therapeutic agents on hepatic metastatic dissemination. Irinotecan monotherapy resulted in a significant 44% reduction in hepatic metastatic mass relative to vehicle controls (P < 0.001), whereas neither oxaliplatin monotherapy nor CEP-7055 monotherapy had significant effects on hepatic metastatic burden compared with vehicle-treated mice (Fig. 4B). The combination of CEP-7055 and irinotecan resulted in a 58% reduction in hepatic metastatic burden relative to vehicle controls (P < 0.001) but, more importantly, a significant 52% reduction in hepatic metastatic burden relative to that achieved with irinotecan monotherapy (P < 0.01). The administration of CEP-7055 and oxaliplatin resulted in a 43% reduction in hepatic metastatic burden relative to controls (P < 0.05) and showed a trend for an improvement relative to oxaliplatin monotherapy (P = 0.08). These data indicate that combination therapies of CEP-7055 and irinotecan, and to a lesser extent oxaliplatin, show significant reductions in hepatic metastatic burden in this model relative to those achieved by monotherapy with traditional cytotoxic agents. Moreover, the combination therapy of oxaliplatin plus CEP-7055 was well tolerated with no signs of morbidity, and the combination of irinotecan plus CEP-7055 resulted in body weight loss, which achieved significance (P ≤ 0.01) only on day 8 relative to irinotecan monotherapy, with animals regaining weight by day 12 (Fig. 4C).

**Table 1. Incidence of cerebral hemorrhage and cerebral edema in orthotopic human glioma-bearing mice treated with CEP-7055 alone or in combination with temozolomide**

<table>
<thead>
<tr>
<th>Treatment group (no. mice/group)</th>
<th>% Mice with cerebral hemorrhage</th>
<th>% Mice with cerebral edema (scores 1–4)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (15)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>CEP-7055: 23.8 mg/kg/dose (14)</td>
<td>71</td>
<td>14</td>
</tr>
<tr>
<td>CEP-7055: 59 mg/kg/dose (15)</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>CEP-7055: 23.8 mg/kg/dose + temozolomide (15)</td>
<td>0*</td>
<td>100</td>
</tr>
<tr>
<td>CEP-7055: 59 mg/kg/dose + temozolomide (15)</td>
<td>0*</td>
<td>100</td>
</tr>
<tr>
<td>Temozolomide: 68 mg/kg/dose × 5 d (15)</td>
<td>7*</td>
<td>93</td>
</tr>
</tbody>
</table>

*Cerebral edema scoring in the brain is further detailed in Materials and Methods. The extent and degree of cerebral edema was assessed as follows: 0, no visible tumor; 1, edematous lesions confined to surrounding tumor at implantation site; 2, edematous lesions spread into brain tissue adjacent to tumor implantation site; 3, edematous lesions identified in 3 of 4 (75%) quadrants of brain tissue; 4, edematous lesions identified in 4 of 4 (100%) quadrants of brain tissue.

1P < 0.05, Mann Whitney rank-sum test for incidence of hemorrhagic relative to vehicle controls.

Discussion

Currently available treatment options for glioblastoma multiforme and colorectal cancer have made improvements in the quality-of-life for patients with these cancers; however, they are still limited in the overall objective response rates and survival benefit (2, 6, 9, 13, 14). Based on the highly vascularized nature of these tumors, novel therapeutic strategies employing antiangiogenic agents in combination with traditional cytotoxic agents are being evaluated for treating these cancers. Antiangiogenic therapies directed against the VEGF-VEGFR kinase axes through a variety of approaches have been a promising and well-validated therapeutic approach under active evaluation for multiple solid tumors, including glioblastoma multiforme and colorectal carcinoma (18, 23–25). The use of bevacizumab in patients with colorectal cancer in combination with the irinotecan and 5-fluorouracil/leucovorin regimen resulted in significant clinical benefit by increasing survival, objective response, and time to progression of disease (37, 38). This observed increase in the survival rate as well as the improvement of other biomarkers indicating a clinical benefit provided clinical proof-of-concept for the use of antiangiogenic inhibitors targeting the VEGF-VEGFR axis in combination with standard chemotherapy for the treatment of patients with colorectal cancer (24, 37, 38).

CEP-7055, a novel, fully synthetic, orally active N,N-dimethylglycine ester of CEP-5214, a C3-(isopropyl-methoxy)–fused pyrrolocarbazole with potent pan-VEGFR kinase inhibitory activity, has recently completed phase I clinical trials in cancer patients. The parent compound CEP-5214 is a low nanomolar, orally active inhibitor of all three VEGFR kinase receptor subtypes (VEGFR1/FLT-1 IC<sub>50</sub>, 16 nmol/L; VEGFR2/KDR IC<sub>50</sub>, 8 nmol/L; and VEGFR3/FLT-4 IC<sub>50</sub>, 4 nmol/L), displays potent antiangiogenic activity in multiple in vitro and ex vivo models, and shows oral antitumor efficacy against a variety of rodent and human s.c. tumor xenograft models.
(A375, ASPC-1, Calu-6, HT-29, RENCA, and U87MG) in athymic nude mice and on retinal vascular permeability and leakage in a rat model of diabetes in the absence of apparent morbidity or toxicity (24, 28, 39). Its ester derivative, CEP-7055, was prepared to increase aqueous solubility and to facilitate oral delivery. We have shown previously that the magnitude of human and rodent tumor xenograft growth inhibition observed with chronic administration of CEP-7055 at 23.8 to 47.6 mg/kg/doses across multiple organ-specific tumor types is comparable with or lower than that observed with chronic oral administration of several VEGFR kinase inhibitors currently under clinical evaluation (23–25, 28). The present studies expand on these findings and show distinct and significant antitumor effects of CEP-7055 administration in combination with clinically relevant chemotherapeutic agents in two orthotopic tumor models of primary and metastatic disease relative to both vehicle-treated animals and, more importantly, those given these cytotoxic agents as standard monotherapy.

The U87MG glioblastoma multiforme model is a highly vascularized and invasive tumor used in numerous studies to evaluate antiangiogenic therapies (40, 41). Earlier studies in our laboratory showed that these tumor xenografts produce abundant amounts of angiogenic factors in situ, including VEGF-A, VEGF-C, neuropilin, basic fibroblast growth factor, platelet-derived growth factor, Ang-1, Ang-2, and their corresponding

**Figure 4.** Effects of CEP-7055 alone and in combination with irinotecan or oxaliplatin on primary colon tumors and hepatic metastatic burden in the CT-26 spleen-implanted metastatic colon carcinoma model in BALB/c mice. Two days following recovery from surgery as detailed in Materials and Methods, mice were randomized into the following treatment groups (n = 10): 1% aqueous acetic acid vehicle, CEP-7055 monotherapy (23.8 mg/kg/dose p.o. b.i.d.), irinotecan 20 mg/kg/dose i.p. once daily for 5 d in 0.9% sterile saline, CEP-7055 in combination with irinotecan dosed as described above, oxaliplatin 10 mg/kg/dose i.v. in 5% dextrose, and CEP-7055 in combination with oxaliplatin dosed as described above. A, primary spleen-implanted colon tumor mass in the CT-26 colon carcinoma model in BALB/c mice following 18 d of treatments. Statistical analyses were conducted using the Mann-Whitney rank-sum test. *, P < 0.05; **, P < 0.001, relative to vehicle-treated controls; A, P < 0.01, combination of CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) and irinotecan (20 mg/kg/dose i.p. q.d. × 5 d) treatment relative to irinotecan monotherapy; B, no statistically significant difference between the combination of CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) and oxaliplatin (10 mg/kg/dose i.v.) treatment relative to oxaliplatin monotherapy. B, hepatic metastatic burden in the CT-26 colon carcinoma model in BALB/c mice following 18 d of treatments. Statistical analyses of metastatic burden were conducted using the Mann-Whitney rank-sum test. *, P < 0.05; **, P < 0.001, relative to vehicle-treated controls; A, P < 0.01, combination of CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) and irinotecan (20 mg/kg/dose i.p. q.d. × 5 d) treatment relative to irinotecan monotherapy; B, P = 0.08, combination of CEP-7055 (23.8 mg/kg/dose p.o. b.i.d.) and oxaliplatin (10 mg/kg/dose i.v.) treatment relative to oxaliplatin monotherapy. C, effects of CEP-7055 alone and in combination with irinotecan and oxaliplatin on body weights of BALB/c mice with spleen-implanted murine CT-26 colon carcinoma cells. Body weight measurements of BALB/c mice implanted with CT-26 colon carcinoma cells over 18 d of treatments. Bars, SE. Statistical analyses were conducted using the Mann-Whitney rank-sum test. ***, P < 0.001 relative to vehicle-treated controls.
receptors, and that s.c. implants of these tumors are growth inhibited in response to oral administration of CEP-7055 (28). Moreover, earlier studies examined the antitumor efficacy of oral CEP-5214, the active moiety of CEP-7055 (10 mg/kg/dose equivalents p.o. b.i.d.), and the alkylating agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (26 mg/kg/dose i.p. single dose), on s.c. U87MG human glioblastoma xenografts (36). These studies revealed that CEP-5214 and 1,3-bis(2-chloroethyl)-1-nitrosourea combination treatment was well tolerated and resulted in complete tumor regressions in 33% of cases and partial tumor regressions in 67% of cases versus 25% incidence of partial regressions with CEP-5214 monotherapy and 80% incidence of partial regressions with 1,3-bis(2-chloroethyl)-1-nitrosourea monotherapy (36). These observations provided a basis, in part, for the series of studies reported here with optimal effective dosing regimens of CEP-7055 and temozolomide in a more clinically meaningful orthotopic model of human glioblastoma multiforme with the most critical end point being effects of combination therapy on median survival relative to temozolomide monotherapy.

Collectively, data generated in two independent studies of $\sim$400-day in-life duration in the human orthotopic U87MG glioblastoma model administering CEP-7055 and a clinically relevant dosing regimen of temozolomide in combination indicate both a significant survival benefit of CEP-7055 in combination with temozolomide compared with temozolomide monotherapy and a marked reduction in apparent neurologic deficit (abnormal behavioral responses) and brain edema/hemorrhagic lesions with chronic CEP-7055 administration alone and when given in combination with temozolomide. Further, no distinct drug-associated morbidity or toxicities were observed with chronic administration of CEP-7055 alone or in combination with several 56-day cycles of temozolomide oral administration over the in-life phase of these studies. The reductions in intratumoral microvessel density observed in these studies with CEP-7055 administration are consistent with earlier observations and the mechanism of action, at least in part, of CEP-7055 given its pan-VEGFR kinase inhibitor activity and pharmacologic activity in $in$ vitro, $ex$ vivo, and $in$ vivo models of angiogenesis (28, 36). The reductions in brain edema and behavioral impairments with administration of CEP-7055 monotherapy, despite the absence of significant survival benefit or reduction in intracranial tumor mass, are consistent with earlier observations for significant and sustained effects of oral CEP-7055 administration on inhibiting VEGF-induced dermal vascular permeability in rats (ED$_{50}$ 20 mg/kg/dose p.o.; ref. 28) and VEGF-induced retinal vascular permeability in normal and diabetic rats over this same dose range (39). Given the highly angiogenic nature of the VEGF-producing U87MG tumor and the effects of CEP-7055 on reducing tumor microvessel density, the observed reductions in edematous lesions observed in intracranial tumors with CEP-7055 administration alone and in combination with temozolomide are a reasonable experimental outcome in these studies. Similar observations for a reduction in VEGF-mediated cerebral edema have been observed with other VEGFR kinase inhibitors chemically related to CEP-7055 (42).

The CT-26 murine colon carcinoma model of hepatic metastases has been employed in the preclinical evaluation of several antiangiogenic therapies (26, 27). The use of oxaliplatin and irinotecan showed an improvement in the efficacy of 5-fluorouracil/leucovorin in first-line or second-line treatment for colorectal cancer (11–14). Although tumor response rates have reached $\approx$50% on these regimens, the median overall survival is only 18 months (14). A substantial improvement in time to progression, progression-free survival, and overall survival of metastatic colon cancer was observed when these cytotoxic regimens were combined with the humanized anti-VEGF antibody, bevacizumab, in clinical trials (37, 38). Consequently, the identification and evaluation of targeted therapeutic agents for use in combination with these standard-of-care cytotoxic therapies is essential to help manage patients with advanced disease (43). In this regard, the effects of CEP-7055 on primary colon carcinoma growth and hepatic metastatic burden in the CT-26 murine model was evaluated in combination with established dosing regimens of irinotecan or oxaliplatin (11, 12, 32, 35). The administration of CEP-7055 in combination with irinotecan, and to a lesser degree oxaliplatin, showed significant reductions on primary colon carcinoma and hepatic metastatic burden in the CT-26 tumor model relative to irinotecan and oxaliplatin monotherapy. The highly significant efficacy of CEP-7055 and irinotecan in combination versus irinotecan monotherapy against both primary colon tumor mass and hepatic metastatic burden is particularly noteworthy, given the fact that the combination is well tolerated, with significant body weight loss observed on a single day of treatment. These data and the fact that irinotecan is approved as first-line treatment of metastatic colon cancer lend support for evaluating the use of a pan-VEGFR kinase inhibitor, such as CEP-7055, in combination with irinotecan on various dosing schedules in this clinical setting. The combination of CEP-7055 and oxaliplatin also resulted in significant reductions in both primary and hepatic metastatic tumor burden relative to vehicle controls albeit of lesser magnitude than that achieved with CEP-7055 and irinotecan in combination. Additional dose scheduling studies of CEP-7055 and oxaliplatin are warranted particularly given the strong trends observed in these studies for the efficacy of combined administration of CEP-7055 and oxaliplatin on hepatic metastatic burden relative to oxaliplatin monotherapy. The efficacy data obtained and the tolerability of the combination regimens of CEP-7055 and irinotecan and CEP-7055 and oxaliplatin

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in the studies described here suggest that CEP-7055 coadministration may improve the overall therapeutic index of these cytotoxic agents when used in optimized dosing schedules.

The exact mechanism by which CEP-7055 given in combination with temozolomide and irinotecan confers an improvement in survival and antitumor efficacy relative to monotherapy is uncertain. Several studies have shown that combining therapies targeting the VEGF-VEGFR axis with chemotherapy is more effective on primary and metastatic tumor growth and survival than that achieved with monotherapy (44–46). Given the vascular permeabilizing effects of VEGF on the tumor vasculature and the VEGF-mediated abnormalities in tumor vascular morphology, tortuosity, branching patterns, and blood flow (16, 19, 46) relative to the normal vasculature, several possible explanations for the beneficial effects of combining VEGF-VEGFR-targeted therapies with chemotherapy are plausible. One possibility is a “normalization” of the tumor microenvironment and vasculature by VEGF-VEGFR-directed therapies, increasing the sensitivity of the abnormal tumor vasculature to conventional therapies and leaving more phenotypically normal and functional blood vessels intact within solid tumors, resulting in improved perfusion of the tumor with cytotoxic agents (16, 46–48). In addition, it has been shown that anti-VEGF-VEGFR-directed therapies significantly decrease interstitial fluid pressure and restore hydrostatic and oncotic pressure gradients in tumors resulting in significant improvement in tumor perfusion (see refs. 46, 48). Improvements in vascular perfusion and delivery of irinotecan to s.c. colorectal tumor xenografts following anti-VEGF antibody administration have been shown preclinically (49) as well as in phase I clinical studies in colorectal cancer patients given bevacinumab before adjuvant chemoradiation therapy (37). Similarly, administration of the VEGFR2 inhibitor SU5416 followed by temozolomide treatment to tumor-bearing mice resulted in increased temozolomide delivery to the tumor cells in intracerebral (orthotopic) gliomas but not in s.c. glioma xenografts (50). These VEGF-VEGFR-mediated effects on the tumor microenvironment and vasculature facilitating the delivery of cytotoxic agents to tumor cells may explain, in part, the results obtained in the studies reported here with CEP-7055 and temozolomide and irinotecan combination therapies in orthotopic glioma and colorectal carcinoma models, respectively. Studies examining tumor interstitial fluid pressure and vascular perfusion in these orthotropic tumor models in relation to administration schedules of CEP-7055 are necessary to confirm these hypotheses.

In conclusion, the results obtained in aggressive orthotopic models of glioblastoma and colorectal cancer show the efficacy, survival benefit, and tolerability of chronic administration of CEP-7055 in combination with cytotoxic chemotherapeutic agents used clinically for the treatment of these malignancies. These preclinical data lend support for consideration of the potential clinical evaluation of CEP-7055 in combination with standard-of-care therapies in patients with glioblastoma and metastatic colorectal cancer.

References
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The effects of the oral, pan-VEGF-R kinase inhibitor CEP-7055 and chemotherapy in orthotopic models of glioblastoma and colon carcinoma in mice

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